Biochimica et Biophysica Acta 1783 (2008) 2384–2390

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Lunatic fringe protein processing by proprotein convertases may contribute to the short protein half-life in the segmentation clock

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ARTICLE INFO

Article history:
Received 20 May 2008
Received in revised form 10 July 2008
Accepted 11 July 2008
Available online 25 July 2008

Keywords:
Notch
Somitogenesis
Segmentation clock
Lunatic fringe
Glycosyltransferase

ABSTRACT

During vertebrate segmentation, oscillatory activation of Notch signaling is important in the clock that regulates the timing of somitogenesis. In mice, the cyclic activation of NOTCH1 requires the periodic expression of Lunatic fringe (*Lfng*). For LFNG to play a role in the segmentation clock, its cyclic transcription must be coupled with post-translational mechanisms that confer a short protein half-life. LFNG protein is cleaved and released into the extracellular space, and here we examine the hypothesis that this secretion contributes to a short LFNG intracellular half-life, facilitating rapid oscillations within the segmentation clock. We localize N-terminal protein sequences that control the secretory behavior of fringe proteins and find that LFNG processing is promoted by specific proprotein convertases including furin and SPC6. Mutations that alter LFNG processing increase its intracellular half-life without preventing its secretion. These mutations do not affect the specificity of LFNG function in the Notch pathway, thus regulation of protein half-life affects the duration of LFNG activity without altering its function. Finally, the embryonic expression pattern of *Spc6* suggests a role in terminating LFNG activity during somite patterning. These results have important implications for the mechanisms that contribute to the tight control of Notch signaling during vertebrate segmentation.

The Notch signaling pathway is a widely expressed, highly conserved pathway involved in numerous developmental mechanisms [1]. The central events of Notch signaling are straightforward. NOTCH receptor binding to JAGGED and/or DELTALIKE ligands, (SERRATE and DELTA in *Drosophila*) promotes the release of the intracellular receptor domain (NICD) by a series of cleavage events. NICD translocates to the nucleus and promotes transcription of target genes [2]. This pathway can be controlled spatially and temporally through modification of NOTCH receptors by *fringe* family proteins, which in vertebrates include Lunatic, Manic, and Radical fringe (*Lfng*, *Mfng* and *Rfng*) [3,4]. *Fringe* genes encode glycosyltransferases that modulate Notch signaling by modification of the NOTCH extracellular domain [5,6]. FRINGE proteins transfer *N*-acetylglucosamine to fucose on extracellular EGF repeats of NOTCH receptors, but it is not well understood how sugar addition alters the interactions between NOTCH and its ligands [7]. In vitro reconstitution of the NOTCH:ligand interactions utilizing *Drosophila* proteins finds that glycosylation of the receptor enhances its binding to DELTA, but inhibits its binding to SERRATE [8]. In mammalian systems, however, different fringe proteins have distinct effects on Notch signaling, perhaps allowing for context-dependent fine-tuning of Notch signaling (for example [9–11]).

One embryonic process that requires the modulation of Notch signaling by LFNG is vertebrate segmentation [2,12,13]. Notch signaling and *Lfng* expression play multiple roles during somitogenesis. In the posterior presomitic mesoderm (PSM) of many vertebrates, oscillatory NOTCH1 activity is regulated, at least in part, by feedback loops that involve the modulation of NOTCH1 by LFNG. Cyclic activation of NOTCH1 is important in the segmentation clock that times the process of somitogenesis. In the anterior PSM, NOTCH1 is critical for the rostral/caudal (R/C) patterning of somites, and here again LFNG may play key roles in localizing Notch signaling to the appropriate compartment [12,14].

For *Lfng* to play a role in the segmentation clock, its protein activity levels must oscillate with a short period (two hours in the mouse). During chick segmentation, LFNG protein levels, as well as *Lfng* transcript levels, oscillate with a period that matches somite formation, linking LFNG protein activity to the clock [15]. Cyclic *Lfng* expression is regulated transcriptionally [16,17], but little is known about the post-translational mechanisms that contribute to the rapid periodicity of its function in the segmentation clock. The functions of FRINGE proteins within the Notch pathway are cell autonomous [5,10,18–20], but interestingly, both *Drosophila* FRINGE and mouse LFNG protein are cleaved following a conserved dibasic site, and are secreted into the media when expressed in tissue culture cells [3]. This suggests the possibility that LFNG secretion could provide a mechanism to terminate LFNG function in the Notch pathway, facilitating the rapid oscillations of LFNG activity in the segmentation clock.

The sequence of the identified LFNG processing site (RARR in mouse) suggests that the protein may be cleaved by members of the

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doi:10.1016/j.bbamcr.2008.07.009

subtilisin-like proprotein convertase family (SPCs) [3]. This family has nine known members that play diverse roles in the processing and maturation of many substrates including proteases, hormones and growth factors [21]. Seven of these proteins (furin/Pcsk3/SPC1, SPC2/Pcsk2/PC2, SPC3/Pcsk1/PC1/3, SPC4/Pcsk6/PACE4, SPC5/Pcsk4/PC4, SPC6/Pcsk5/PC6, and SPC7/Pcsk7) process their substrates at multi-basic sites with the motif (K/R)XX(K/R), and in many cases this protein processing is required for activation of the substrate. To better understand the post-translational regulation of LFNG activity, we examined the roles of LFNG processing by SPC convertases.

Materials and methods

LFNG mutants

AP-tagged mouse FRINGE coding sequences [3] were ligated into pcDNA3 (Invitrogen). For HA-tagged vectors the FRINGE coding sequences were transferred into a pcDNA3 vector with a C-terminal HA tag. *Rfng* or *Mfng* N-terminal sequences were amplified and RFNG aa 1–59 or MFNG aa 1–54 replaced LFNG aa 1–112 in R/LFNG and M/LFNG respectively. LFNG^{m1} (RARR to AAAA) and LFNG^{m2} (RGRR to AAAA) were created by 2 step PCR based mutagenesis (primer sequences in SI Table 1).

Alkaline phosphatase assays

4×10^4 NIH3T3 cells (grown in DMEM supplemented with 10% FBS, 50 mM glutamine) were plated in 24-well plates and co-transfected 24 h later with 800 ng of AP-fringe plasmid and 200 ng of pSVβgal (Promega) using Lipofectamine 2000 (Invitrogen). After 24 h, media was collected and cells were lysed with 100 µl Passive Lysis Buffer (Promega). 50 µl of the cellular extracts or 50 µl of heat inactivated culture was mixed with 50 µl of AP Assay Reagent A (GenHunter) to determine AP activity following the manufacturer's instructions. AP activity was calculated as (OD_{405}*54)/(Reaction time*Sample Volume) minus the background AP activity of pCDNA3 control and was normalized to β-gal activity levels as a control for transfection efficiency. 30 µl of the cell assayed by mixing with the substrate ONPG, using standard protocols. After incubation at 37 °C until a yellow color was detected, reactions were stopped by addition of 1 M Na_2CO_3, and optical density was measured at 420 nm. For each experiment the percent of AP activity in the media and in the cellular fraction were calculated.

Immunofluorescence

Cells were plated on glass cover slips, transfected as described above and fixed in 8% PFA. Coverslips were incubated with anti-AP antibody (Fitzgerald Industries, 1:100) and anti-GM130 antibody (BD Biosciences, 1:200). Secondary Alexafluor antibodies (594 anti-rabbit and 488 anti-mouse, Invitrogen) were diluted 1:1000. Cells were counterstained with Hoechst dye. Coverslips were mounted with Citifluor and examined with an Olympus 1X81 microscope. Each experiment was performed at least twice and multiple fields of cells were examined to determine intracellular localization.

NOTCH1 signaling assay

An established Notch signaling assay was utilized to assess the effects of fringe proteins on JAGGED1 induced signaling [10]. NIH3T3 cells were plated as described above and transfected with 100 ng of pBOSrNotch1 [22], 100 ng of AP-tagged fringe expression vector or empty APtag4 expression vector, 200 ng of a CBF1-luciferase reporter construct [23], and 200 ng pSVβGal for normalization of transfection efficiency. After 16 h, the cells were co-cultured for 24 h with 1.24×10^6 control L-cells or L-cells stably expressing JAGGED1 [24]. 20 µl of cell lysates were analyzed by luciferase assay (Promega). Luciferase values were normalized to βgal expression (measured as above). Notch-induced activation of CBF1 is expressed as a ratio of normalized luciferase values induced by the JAGGED-expressing cells compared to that obtained with parental L-cells.

Western blot analysis

NIH3T3 cells were plated and transfected as above with expression vectors encoding HA-tagged fringe proteins, and 6 h after transfection the media was changed to DMEM+2% FBS. Expression vectors encoding α_1PDX protease inhibitor [25], SPC1/furin, SPC4, or SPC7 [26], SPC6A or SPC6B (from D. Constam) were co-transfected as indicated. For Fig. 1A, plasmid amounts were: 350 ng LFNG, α_1PDX as indicated, and pcDNA3 to bring up total DNA to 900 ng. For Fig. 1B plasmid amounts were: 300 ng LFNG, 100 ng α_1PDX, 500 ng SPC vector as indicated and pcDNA3 to bring up total DNA to 900 ng. For Fig. 2 a total of 1000 ng of DNA was transfected, either fringe expression vector alone or equal amounts of fringe expression vector, α_1PDX and/or SPC6A expression vector. After incubating cells in DMEM+2% FBS for 24 h, 500 µl of cell media was concentrated to ~100 µl with Microcon columns (Millipore). 11.5 µl of concentrated media were mixed with 2× Laemmli loading buffer, run on a 12% polyacrylamide gel and transferred to an Immobilon membrane (Millipore). The membrane was incubated with an anti-HA antibody (HA-7, 1:1000, Sigma-Aldrich) and analyzed using the ECL System following the manufacturer's protocols (GE Healthcare).

Cycloheximide treatment

2.6×10^5 NIH3T3 cells were plated in a 6-well plate and transfected with 300 ng (LFNG, LFNG^{m1}) or 100 ng (LFNG$^{m1/2}$, R/LFNG) HA-tagged expression vectors using Lipofectamine 2000 (Invitrogen) resulting in similar, low levels of protein expression. After 6 h, transfected cells were split and 1.25×10^5 cells were plated into five wells of a 24-well plate, ensuring that all samples in the time course were transfected with equivalent efficiency. After 16 h, cells were incubated in media with 20 µg/ml cycloheximide (Sigma). Cell extracts were collected at 20-minute intervals, lysed directly in 100 µl Laemmli loading buffer, and 23 µl of each sample/time point was analyzed by Western blot

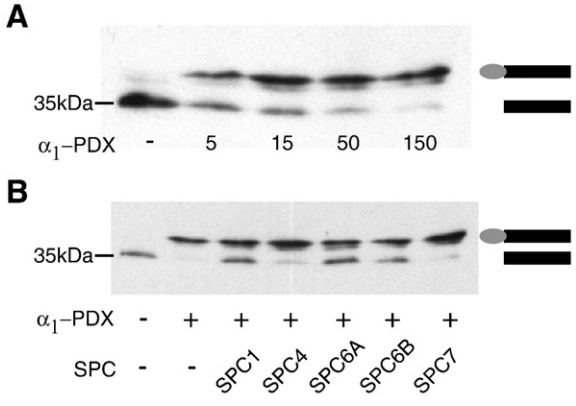

Fig. 1. LFNG is processed by SPC proconvertases. A. Media fractions from NIH3T3 cells transfected with expression vectors encoding wild-type mouse LFNG (LFNG^{wt}) and α_1PDX were analyzed by Western blot (ng of α_1PDX expression vector indicated). Protein species are diagrammed, (black box = mature LFNG, gray oval = LFNG pro region). α_1PDX expression reduces the amount of secreted mature LFNG (35 kDa) and increases the amount of full-length LFNG (43.6 kDa) suggesting a general inhibition of LFNG processing. C. NIH3T3 cells were transfected with LFNG expression vector, α_1PDX expression vector, and SPC protease expression vectors as indicated, and media fractions were analyzed by Western blot. SPC1, SPC6A, and SPC6B efficiently compete with the inhibitor and cleave LFNG, while SPC4 and SPC7 are less efficient.

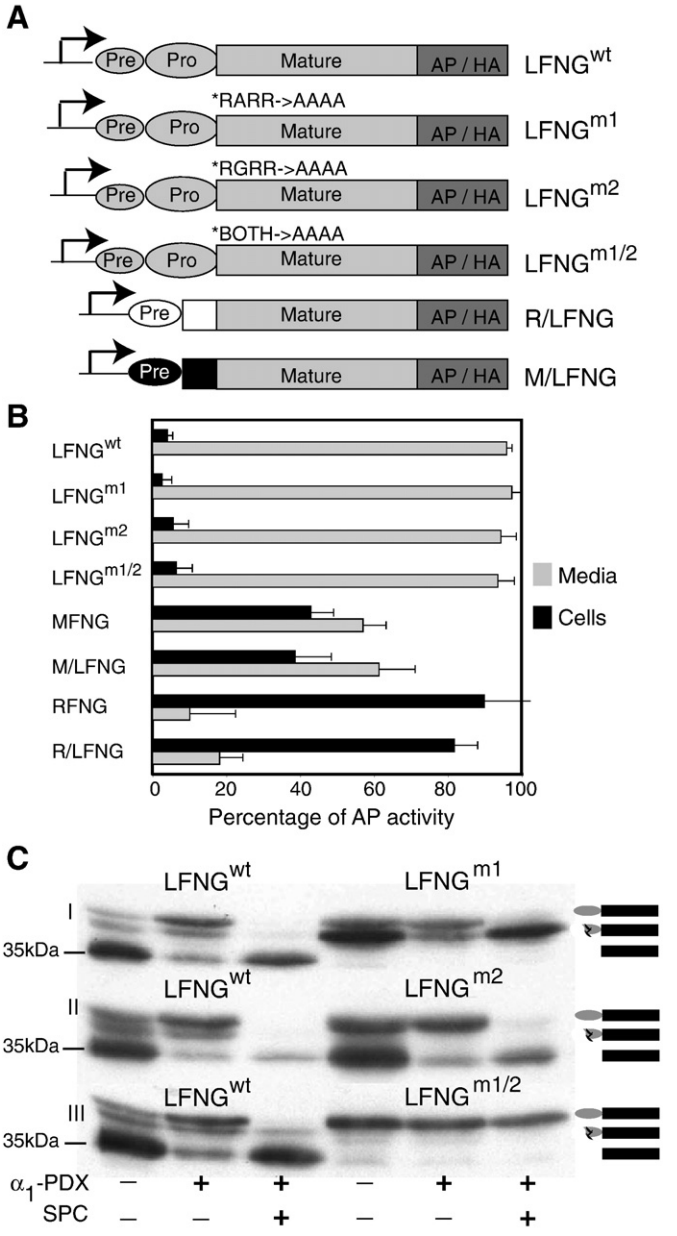

Fig. 2. Variant LFNG proteins have altered secretory behavior. (A) Dibasic sites in mouse LFNG were mutated (*) in constructs LFNG^{m1}, LFNG^{m2}, LFNG$^{m1/2}$. For chimeric fringe proteins the first 112 aa of wild-type LFNG (grey circles) were replaced with either the first 59 aa of RFNG (white circle) or the first 54 aa of MFNG (black circle). Fusion is at LFNG D113 the first conserved amino acid in fringe proteins. (B) Steady-state AP activity was measured in the cell extracts and media of cells transfected with vectors encoding AP-tagged fringe proteins. Results are shown as the fraction of total AP activity in the cellular or media fraction and are the mean±SD of at least three independent experiments. LFNG is found almost entirely in the media, as are the mutated cleavage constructs, while RFNG and R/LFNG are almost entirely retained in the cell. MFNG and M/LFNG are detected at similar levels in both cellular extracts and media. (C) Cells were transfected with HA-tagged LFNG and LFNG^{m1} (I), LFNG^{m2} (II), or LFNG$^{m1/2}$ (III). α_1PDX and/or SPC6A vectors were co-transfected as indicated. HA-tagged proteins in the media were analyzed by Western blot. LFNG is found in the media predominantly as the 35 kDa mature form. Bands corresponding to the predicted full-length protein (43.6 kDa) and a band of intermediate size (40.4 kDa) are also detected (lane 1). α_1PDX blocks the formation of the mature protein and the amount of the full-length protein is increased (lane 2). SPC6A drives production of mature LFNG (lane 3). LFNG^{m1} is detected as either the intermediate or full-length protein when expressed. α_1PDX blocks production of the intermediate LFNG band, which is recovered upon expression of SPC6A (I, lanes 4–6). LFNG^{m2} blocks production of the intermediate band and is secreted as the mature 35 kDa fragment. α_1PDX expression blocks the formation of the mature protein, which is recovered with expression of SPC6A (II, lanes 4–6). LFNG$^{m1/2}$ is found in the media exclusively as the full-length protein (III, lanes 4–6). Secreted protein species are diagrammed.

utilizing Alexafluor anti-mouse 680 secondary antibody (1:20,000, Invitrogen). Blots were imaged and quantitated on a Li-Cor Odyssey. Blots were re-probed using a monoclonal anti-tubulin antibody (1:1000, Sigma). Exponential trend lines were fitted to data points to calculate protein half-lives. Experiments were repeated at least six times and outliers >2×IQR from the median were excluded.

Whole mount in situ hybridization

Wild-type embryos were collected from timed pregnancies with noon of the day of plug identification designated at 0.5 d.p.c. Double RNA *in situ* hybridization using digoxigen and fluorescein-labeled probes was performed as described [16]. Probes: *Mesp2* [27] and *Spc6*, which recognizes both *Spc6A* and *Spc6b* spliceforms [28].

Results

SPC1/furin, SPC6A and SPC6B promote LFNG processing

LFNG cleavage occurs after a conserved RARR sequence, a consensus recognition site for the SPC family of proprotein convertases [3]. To examine whether LFNG processing is reliant on specific SPC proteins, we assessed whether the SPC inhibitor α_1 Antitrypsin Portland (α_1PDX) [25] could inhibit this processing. HA-tagged mouse LFNG (LFNG) was co-expressed with increasing amounts of α_1PDX (Fig. 1A). In the absence of α_1PDX, a 35 kDa LFNG fragment is detected in the media, consistent with the predicted size of the fully processed, mature fragment (34.1 kDa, all calculated sizes include HA tag). The mature fragment is reduced as α_1PDX levels increase, and we observe a corresponding increase in the release of a 43.6 kDa fragment, which is the predicted size for full-length, unprocessed LFNG protein (43 kDa). These data indicate that inhibition of SPC proteins interferes with LFNG processing at the conserved dibasic cleavage site, but suggest that proteolytic processing of LFNG is not absolutely required for its secretion from tissue culture cells. Even in the absence of α_1PDX, the 35 kDa mature fragment is rarely observed in cellular lysates (and then only on long exposures of overloaded gels), suggesting that unlike the full-length protein, the processed 35 kDa form of LFNG cannot accumulate inside cells, possibly due to its rapid secretion (data not shown).

α_1PDX is reported as a specific inhibitor of furin and SPC6 [25], suggesting that only a subset of SPC family members may efficiently process LFNG. To test this idea, LFNG was expressed along with intermediate levels of α_1PDX expression vector to inhibit endogenous cleavage. Co-transfection of expression vectors encoding different SPC family members assessed which convertases could efficiently process LFNG protein. Expression of SPC1/furin, SPC6A, or SPC6B results in the recovery of the processed LFNG fragment. In contrast, expression of either SPC4 or SPC7 results in limited LFNG processing (Fig. 1B). These results confirm that furin, SPC6A and SPC6B are able to efficiently process LFNG protein, and indicate specificity among SPC family members in their recognition of LFNG as a substrate.

N-terminal sequences regulate the secretory behavior of fringe family proteins

Golgi retention of glycosyltransferases remains poorly understood, but in many glycosyltransferases, sequences at the N-terminus are important in protein localization and/or secretion [29]. The mammalian fringe proteins exhibit distinct Golgi retention and secretion behaviors when expressed in tissue culture cells, and the regulation of these different behaviors is unknown. LFNG is secreted as a processed, mature fragment. MFNG is secreted more slowly, while RFNG is a Golgi resident protein [3]. We assessed the secretory behavior of AP-tagged

mouse fringe proteins by tracking the steady-state levels of AP activity in the cellular extract and media fractions of tissue culture cells (Fig. 2A, B). LFNG protein is observed primarily in the media fraction, reflecting its secretion. In contrast, RFNG remains confined to the cellular fraction, while MFNG protein is found in both fractions, suggesting it is secreted more slowly than LFNG (Fig. 2B). To localize the protein sequences regulating the secretory behavior of the fringe family proteins, chimeric fringe proteins were engineered, replacing the N-terminus of LFNG (including the dibasic cleavage site) with the N-terminal domain of either RFNG (R/LFNG) or MFNG (M/LFNG) (Fig. 2A). In both these chimeric proteins, we find that secretory behavior is controlled by the protein sequences found at the N-terminus. Like RFNG, R/LFNG is detected mostly in cell extracts, thus the RFNG N-terminus is sufficient to confer Golgi retention on the LFNG mature fragment. The M/LFNG protein also mirrors the steady-state levels of MFNG, being detected in both the cell extracts and media at similar levels to MFNG (Fig. 2B). Regardless of their effects on protein secretion, all LFNG variant proteins localize to the Golgi, as previously described (Fig. S1) [10]. Thus, the secretion of LFNG protein relies on sequences found at the N-terminus of the protein, and the N-terminal sequences of other FRINGE proteins are sufficient to properly regulate Golgi retention and or secretion.

SPC family proteases recognize two dibasic cleavage sites in LFNG, but protein processing is not required for secretion

To examine the impacts of the cleavage site on mouse LFNG protein secretion, the conserved RARR cleavage site was mutated to AAAA ($LFNG^{m1}$), and protein secretion and processing were examined. Like LFNG, $LFNG^{m1}$ is found largely in the media fraction, indicating that protein processing at the primary cleavage site is not required for protein secretion (Fig. 2B). Expression of HA-tagged LFNG protein results in the secretion of the 35 kDa, fully processed fragment. Longer exposure reveals small amounts of a 43.6 kDa band, presumably corresponding to the full-length LFNG protein, and an intermediate 40.4 kDa band. Co-expression of α_1PDX reduces the secretion of the fully processed 35 kDa band, and increases the amounts of the 40.4 and 43.6 kDa bands, while overexpression of SPC6 results in the secretion of only the fully processed LFNG mature fragment (Fig. 2C). Mutation of the major processing site ($LFNG^{m1}$) leads to the loss of the 35 kDa band, confirming that the RARR sequence is required for processing at this site. $LFNG^{m1}$ protein is secreted as a predominant 40.4 kDa band and co-expression of α_1PDX causes a reduction in this band and an increase of the full-length 43.6 kDa fragment. Overexpression of SPC6 results in the secretion of only the 40.4 kDa band, indicating that this fragment arises from the processing of full-length LFNG by an SPC proconvertase at a site N-terminal to the previously described site (Fig. 2C). We hypothesized that this fragment might result from cleavage of LFNG after the dibasic RGRR site found at amino acid 40 and mutated that sequence to AAAA either by itself ($LFNG^{m2}$) or in combination with the RARR to AAAA mutation described above ($LFNG^{m1/2}$). Both $LFNG^{m2}$ and $LFNG^{m1/2}$ are found predominantly in the media fraction of transfected cells (Fig. 2B). As predicted, the RGRR mutation ($LFNG^{m2}$) causes the loss of the intermediate 40.4 kDa fragment, confirming that this band results from LFNG processing after the RGRR sequence. $LFNG^{m1/2}$ is found in the media only as a full-length 43.6 kDa band, confirming that protein processing by SPC proprotein convertases is not required for the release of LFNG protein from tissue culture cells (Fig. 2C). Together these data indicate that LFNG is cleaved by SPC proprotein convertases at two sites in the protein. The originally described RARR site appears to constitute the primary cleavage site, but the RGRR site can be utilized. However, neither of these processing events is necessary for the secretion of LFNG from tissue culture cells.

Mutation of SPC processing sites increases the intracellular half-life of the LFNG protein

We find that LFNG processing is not a prerequisite for secretion, however, mutations that interfere with LFNG processing would affect the protein's intracellular half-life if the mature, processed peptide is secreted more efficiently than the full-length protein. To test this idea, we measured the intracellular half-life of mouse LFNG and of LFNG mutant proteins. Cells expressing LFNG, $LFNG^{m1}$, $LFNG^{m1/2}$, or R/LFNG were treated with cycloheximide to inhibit protein translation, and the amount of protein in the cellular fraction was quantified over time (Fig. 3). Under these conditions, we calculate an intracellular half-life for LFNG of 70 min, consistent with rapid turnover in the segmentation clock. Mutation of the primary or both the primary and secondary SPC processing sites causes an increase in intracellular half-life, with both $LFNG^{m1}$ and $LFNG^{m1/2}$ having calculated half-lives of 97 min. Tethering of the LFNG protein in the Golgi further increases the intracellular half-life with a calculated half-life of 126 min for the R/LFNG chimeric protein. These findings suggest that cleavage of LFNG by SPC family convertases may influence the duration of its activity by modulating the rate of LFNG secretion, and thus altering the protein's intracellular half-life.

Alterations of LFNG intracellular half-life do not affect the specificity of its function in the Notch signaling pathway

Different fringe family proteins exhibit distinct effects on Notch signaling depending on the fringe and ligand involved [9]. It is likely that the specificity of fringe activity maps to the catalytic domain of the protein, however, it is possible that mutations that alter LFNG processing and secretion could alter its function in the Notch pathway by changing the secretory behavior of the protein in question (i.e., R/

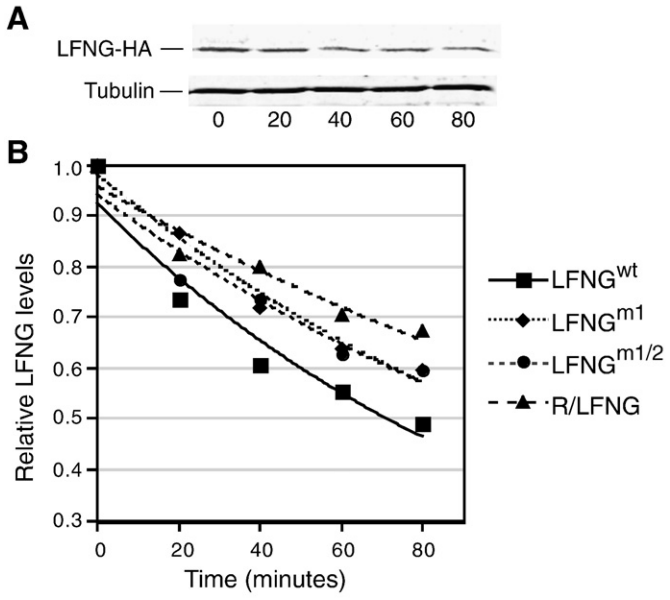

Fig. 3. Mutations affecting LFNG processing result in an increased intracellular protein half-life. (A) NIH3T3 cells expressing HA-tagged mouse LFNG proteins were treated with cycloheximide for an 80-minute time course. Cellular extracts were analyzed by Western blot. A representative LFNG time course is shown indicating that LFNG protein levels decrease over time (indicated in minutes). Tubulin is used as a loading control. (B) Protein concentration in cellular extracts was quantified and normalized to tubulin concentration. Relative protein concentration is shown over time (initial timepoint set to 1). Exponential trend lines were fitted to data points to calculate the intracellular protein half-lives. LFNG has a calculated half-life of 70 min, while $LFNG^{m1}$ and $LFNG^{m1/2}$ have intracellular half-lives of 97 min. The R/LFNG half-life is 126 min.

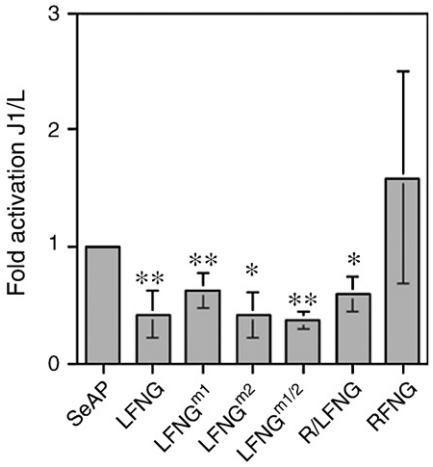

Fig. 4. LFNG variant proteins modify Notch signaling with an LFNG-like activity. NIH3T3 cells were co-transfected with Notch1 expression vector and expression vectors encoding either secreted AP (SeAP) or an AP-tagged mouse fringe protein, along with a CBF1-luciferase reporter and pSVβgal. Transfected cells were co-cultured with cells expressing JAGGED1 or parental LTK− cells. Luciferase values were normalized to βgal values to control for transfection efficiency and are expressed as fold activation reflecting relative luciferase units (RLU) induced by JAGGED1 expressing cells over the RLUs obtained with parental L-cells. (JAGGED1-induction of Notch1 in the absence of fringe was set to 1). Expression of LFNG, LFNG^{m1}, LFNG^{m2}, LFNG$^{m1/2}$, and R/LFNG significantly reduce JAGGED1-induced activation of Notch signaling (*$p<0.02$, **$p<0.005$) while RFNG potentiates JAGGED1-induced Notch1 activation. Error bars represent the standard deviation of the mean from at least three independent experiments performed in triplicate.

LFNG could exhibit the enzymatic activity of RFNG rather than LFNG). To address this question, we assessed whether mutations that affect the secretory behavior of mouse LFNG protein affect the specificity of its activity in the Notch pathway using an established Notch1 signaling assay. As previously reported, we find that expression of LFNG in the signal receiving cell inhibits the ability of JAGGED1 to activate signaling through NOTCH1, while expression of RFNG enhances JAGGED1-induced signaling (Fig. 4 and Ref. [9]). We further find that LFNG^{m1}, LFNG^{m2}, LFNG$^{m1/2}$, and R/LFNG all inhibit JAGGED1-induced signaling to a similar extent as observed for LFNG, thus these mutations alter LFNG processing and half-life without appearing to change its function in the Notch pathway (Fig. 4). These results predict that mutations affecting protein processing will affect the duration, but not the nature of LFNG activity.

The expression pattern of Spc6 suggests a role in clearance of LFNG from maturing somites

Lfng expression during embryonic segmentation is complex. In the posterior PSM, *Lfng* expression is cyclic, and linked to the segmentation clock. In the anterior PSM, *Lfng* expression is restricted to the rostral half of presomite S-1 where it plays important roles in the rostral/caudal patterning of presomites as they mature [12,14]. While *Spc1*/*furin* expression is reported to be ubiquitous during rat embryogenesis [30], specific expression of *Spc6* has been reported in the PSM of developing mouse embryos [28,31]. To assess the potential functional overlap between SPC6 and LFNG during embryonic segmentation, *Spc6* expression was localized in the developing mouse embryo by comparison to that of *Mesp2*, a robust marker of the anterior presomite compartment. Our previously published data shows that *Lfng* and *Mesp2* expression completely overlap in the presumptive rostral compartment of the presomite S-1 [16]. During embryonic segmentation, *Spc6* expression localizes immediately anterior to *Mesp2* in S0, the somite that will next bud from the PSM (Fig. 5A). The expression domains of *Spc6* and *Mesp2* do not overlap, instead a clear border is maintained between the anterior compartment of somite S-1, where *Mesp2* and *Lfng* are co-expressed, and somite S0 where *Spc6* expression is initiated. *Spc6* is also expressed in the most recently formed somite (S1), initially throughout the somite and resolving into a graded expression pattern with higher expression in the rostral somite compartment (Fig. 5B). Thus *Spc6* is expressed exclusively in cells that previously expressed *Lfng*, but which have

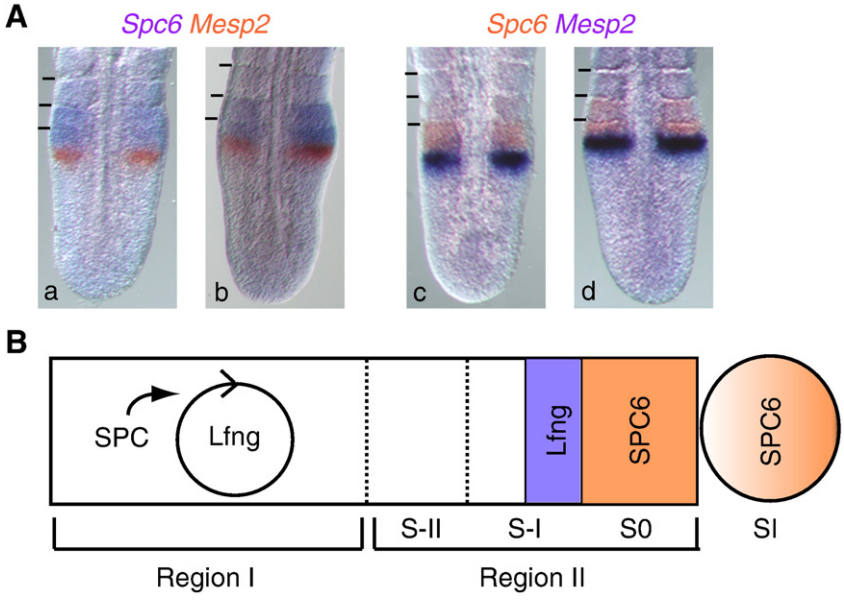

Fig. 5. SPC6 expression patterns suggest a role in clearing LFNG from maturing somites. (A) Whole mount RNA *in situ* hybridization of 10.5 d.p.c. mouse embryos comparing the expression patterns of *Mesp2* and *Spc6*. (a, b) *Spc6* signal is purple, and *Mesp2* signal is orange. (c, d) *Spc6* signal is orange, and *Mesp2* signal is purple. The expression of *Mesp2* does not overlap with that of *Spc6*, forming a clear border at the boundary between presomites S0 and S-1. *Spc6* is also expressed in the newly formed, mature somite S1 either throughout the somite (a, c) or in a graded manner with highest expression in the rostral half of the somite (b, d). Somite borders are indicated by short lines. (B) Schematic of the PSM. In the posterior PSM, LFNG expression is cyclic, and the protein may be cleaved by SPC proconvertases, contributing to its short intracellular half-life. In the anterior PSM, where presomites are patterned, the expression domains of *Lfng* and *Spc6* do not overlap, suggesting that *Spc6* may serve to help cleave and clear any remaining LFNG from the maturing somites, especially from the rostral compartment of S0.

recently downregulated that expression. This supports a possible role for SPC6 in the processing and secretion of residual LFNG protein in somite S0, ensuring that LFNG activity is rapidly inactivated once *Lfng* transcription ceases.

Discussion

For LFNG to function in the segmentation clock or during R/C somite patterning, there must exist post-translational mechanisms that confer a short half-life to its activity in the Notch pathway. We proposed that protein processing by SPCs promotes the secretion and subsequent inactivation of the mature LFNG fragment and that the rapid release of the protein into the extracellular space acts in combination with the cyclic transcription of *Lfng* to facilitate its activity in the segmentation clock and to refine and terminate the functions of LFNG during R/C somite patterning. This would represent a novel mechanism for the regulation of Notch activity allowing tight temporal and spatial modulation of Notch signaling during somitogenesis.

We localized N-terminal sequences that regulate the secretory behavior of the mammalian fringe family proteins, and find that these sequences are sufficient to direct protein processing and/or secretion in a fringe specific manner (Fig. 2). This indicates that, like many glycosyltransferases, sequences at the N-terminus, including putative type II transmembrane domains, are important in fringe localization and/or secretion [29]. Examining the regulation of LFNG processing and secretion, we found that furin and SPC6 can efficiently process LFNG, and identify two distinct sites in LFNG that can be processed by SPC proconvertases (Figs. 1, 2C).

Processing by SPC family proteases is conserved between *Drosophila* FRINGE and mouse LFNG, but the functional roles of LFNG processing have been undetermined. The original descriptions of the *Drosophila* and vertebrate fringes suggested that protein processing might be required to create active, mature protein [3,4,19,32]. However, we find that mutations that block LFNG processing ($LFNG^{m1/2}$), or which tether it in the Golgi (R/LFNG) do not alter activity of the protein in the Notch receiving cell (Fig. 4). Thus, uncleaved protein is active, and has the same specificity in the Notch pathway as wild-type LFNG, and the function of SPC processing does not relate to protein activation.

We propose instead that the secretion of LFNG from the cell acts as a mechanism to terminate LFNG glycosyltransferase activity in the Notch signal receiving cell. This would represent a novel mechanism for the regulation of Notch activity allowing tight temporal and spatial modulation of Notch signaling during somitogenesis. Cleavage and secretion of some glycosyltransferases is proposed to be a general mechanism of turnover [33,34]. For example, the secretion of the ST6Gal I isoform is suggested to limit the sialylation activity of the enzyme [35]. The function of LFNG in the segmentation clock offers an example of a glycosyltransferase which is cleaved and secreted and whose activity must be modulated temporally, providing a situation where the regulated turnover of a glycosyltransferase may indeed be functional *in vivo*.

In the segmentation clock, LFNG has been proposed to cyclically inhibit Notch signaling through its modifications of the NOTCH1 receptor in the Golgi [36]. *Lfng* transcription is periodically inhibited by another Notch target *Hes7* [37]. The cyclic transcription of *Lfng* in combination with its processing by SPC proconvertases and secretion may function to regulate LFNG protein levels, facilitating the oscillations of LFNG activity within the segmentation clock. One intriguing implication of this data would be the idea that processing by SPC proteins plays important roles in clearing LFNG from the cell, influencing the duration of its activity post-translationally. Supporting this idea, we find that mutating the SPC processing sites in LFNG extends its intracellular half-life from 70 to 97 min, presumably by decreasing the secretion rate of the protein (Fig. 3). Experiments that prolong the half-life of HES7 by 8 min in mouse embryos disrupt somitogenesis [38], thus we propose that this alteration in LFNG protein activity half-life would almost certainly affect clock function.

In the anterior PSM, we propose that LFNG activity is required in somite S-1 to modulate Notch signaling during R/C somite patterning, and that LFNG protein is then secreted from cells to terminate that function. The expression pattern we define for SPC6 supports a possible role for that protein specifically in regulating LFNG activity during R/C somite patterning (Fig. 5). We hypothesize that the expression of SPC6 in somites S0 and S1 would promote the rapid cleavage and secretion of any residual LFNG protein in those regions, preventing unwanted LFNG activity. SPC6 has been deleted in mice, and the best described allele results in embryonic death before gastrulation [39]. More recently, this gene has been conditionally inactivated in the mouse epiblast, resulting in altered anterior/posterior patterning, extra thoracic and lumbar vertebrae, uneven rib attachments, and loss of tail structures, among other phenotypes. The authors also find that GDF11 is an *in vivo* substrate of SPC6 [40]. Additional analysis will be required to determine whether LFNG is an *in vivo* substrate of SPC6, and whether loss of LFNG protein processing contributes to the *Spc6* knockout phenotype.

LFNG cleavage in the posterior PSM, during clock function must be regulated by a distinct SPC family member, as we detect no SPC6 expression in this region. Furin, which we show can efficiently process LFNG (Fig. 1), is relatively ubiquitously expressed during embryogenesis, and targeted deletion of this protein causes irregular somitogenesis [41]. Interestingly, SPC6B and furin have been shown to target the same substrates, like BMP4, and are both specifically inhibited by α_1PDX [25,42]. However, SPC6B does not complement the defects seen in SPC1/furin null mice, and SPC1/furin and SPC6 have been shown to localize to different compartments of the Golgi network suggesting similar, but distinct activities [41,43]. This raises the intriguing possibility that different SPC proteases may cleave LFNG with greater or lesser efficiency, functionally creating subtle changes in half-life that may play important roles in the post-translational modulation of LFNG function.

One unaddressed question in fringe biology is the possibility that LFNG may in fact play some active role in the extracellular space. Extracellular roles have been suggested for mammalian fringe proteins, although the mechanisms behind these findings have not been elucidated [32,44,45]. Indeed, extracellular functions have been found for other glycosyltransferases. For example, secreted glycosyltransferases have been hypothesized to have lectin or adhesion functions in the intercellular space [33]. In another case a secreted glycosyltransferase (GnT-V) has been found to possess an independent, extracellular signaling function that does not require its transferase activity [46]. It is unclear at this time whether LFNG plays a role in the extracellular space and what this role might be.

To date it has been difficult to address the functional significance of LFNG processing and secretion *in vivo*. The results reported here define a panel of LFNG mutations that differentially affect protein processing, intracellular half-life, and secretion. In the future, introduction of these mutations into the endogenous *Lfng* locus will allow further dissection of the post-translational regulation of LFNG by SPC proteases, and will address questions of how LFNG processing and secretion affect its functions within, and perhaps outside of, the Notch signaling pathway.

Acknowledgements

We thank G. Weinmaster, D. Constam, and G. Thomas for reagents. We thank T. Vogt and members of his lab for discussion and support during the genesis of this work, and acknowledge A. Freidman for his initial observations of SPC-mediated cleavage of LFNG. We thank members of the Cole lab and Jeff Thompson for the reading of the

manuscript. This work was supported in part by a grant from the American Cancer Society, Ohio Division.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.07.009.

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